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Citation	Hong Kong Medical Journal, 2015, v. 21 n. suppl. 4, p. 39-44
Issued Date	2015
URL	http://hdl.handle.net/10722/232044
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Modulation of cytokine responses by adrenomedullin and adrenomedullin binding protein-1 in macrophages: a novel pathway in sepsis

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KEY MESSAGES

1. Adrenomedullin augments the production of interleukin-10, a cytokine that limits inflammation.
2. Interferon- γ , a cytokine that is increased in inflammation, downregulates the receptor and binding protein of adrenomedullin.
3. Cyclic AMP mediates the increased expression of adrenomedullin, interleukin-6, and interleukin-10 in response to endotoxin.
4. The production of interleukin-6 is mediated by p38-mitogen-activated protein kinase (MAPK), p42/44-MAPK, protein kinase C, and protein

kinase K, whereas that of interleukin-10 is mediated by p38-MAPK and protein kinase K.

5. These downstream pathways could be targets for therapeutic intervention.

Hong Kong Med J 2015;21(Suppl 4):S39-44

RFCID project number: 05050082

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Introduction

Polymicrobial sepsis is a life-threatening disorder. Patients die of septic shock and multiple organ failure caused by lipopolysaccharide (LPS) and other bacterial products. A hyperdynamic phase is followed by shock and circulatory collapse. Myocardial dysfunction frequently accompanies severe sepsis and septic shock secondary to circulating depressant factors, including tumour necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β). Macrophages produce proinflammatory cytokines including TNF- α , IL-1 β , and IL-6 that lead to tissue injury.

Adrenomedullin (AM) is a vasorelaxant peptide originally isolated from the adrenal medulla. It relaxes vascular smooth muscle cells through the elevation of intracellular cyclic adenosine 3'-5'-monophosphate (cAMP).¹ It also acts on endothelial cells by activating adenylyl cyclase and nitric oxide synthase, resulting in dilation of blood vessels. The macrophage produces AM in inflammation and sepsis. Transgenic mice over-expressing AM are resistant to septicaemic shock. AM markedly increased IL-6 production in both resting and LPS-stimulated macrophages, but significantly suppressed LPS-induced TNF- α secretion.² IL-6 and IL-10 can inhibit the production of pro-inflammatory cytokines such as TNF- α and IL-1. These results suggest that AM may play an important role as an anti-inflammatory regulator

of the inflammatory response in the macrophages, at least partly, via its effect on production of inflammatory cytokines.

Elevated levels of AM play a major role in initiating the hyperdynamic response during the early stage of sepsis, yet transition to the late, hypodynamic phase occurs, despite continued high circulating levels of AM.³ A specific AM binding protein-1 (AMBP-1) carries AM in human plasma and is complement factor H.⁴ The decreased vascular response to AM in advanced sepsis is related to a decrease in AMBP-1. Administration of AM/AMBP-1 to septic animals prevents the drop in blood pressure and reduces mortality. AM and AMBP-1 in combination down-regulate pro-inflammatory cytokines in septic animals, and suppress LPS-induced TNF- α expression and release from the macrophages.⁵

The interaction between AM and AMBP-1 has opened a new avenue for research into the transition from the hyperdynamic to the hypodynamic phase of sepsis. AM has been reported to induce its effect through the cAMP-, Ca²⁺, or mitogen-activating protein kinase (MAPK) mediated pathway. Expression of AMBP-1 is detectable in both monocytes and macrophages, and AMBP-1 given in conjunction with AM further raises AM-induced cAMP production. Nonetheless, little is known about the regulation of AMBP-1, the cause of reduced AMBP-1 production in sepsis, or the regulatory

pathways involved to elicit the physiological actions of AM and AMBP-1.

The study aimed to (1) investigate the expression of AM, AMBP-1 and AM receptor proteins in the LPS-induced inflammatory response in a rat alveolar macrophage cell line; (2) examine the effect of AM and AMBP-1 on LPS-induced inflammatory cytokine production in macrophages and determine whether feedback loops or desensitisation of AM receptors affect the production of AM and AMBP-1; and (3) identify a potential role of second messenger-dependent kinases in the regulation of AM and AMBP-1 expression and AM-induced cytokine responses in LPS-stimulated macrophages.

Methods

This study was conducted from October 2006 to March 2008. NR8383 rat macrophages were stimulated by IFN- γ , TNF- α , IL-6, and IL-10 with or without LPS (at 1 to 1000 ng/mL) for 6 and 24 hours.^{2,6} Levels of AM, AMBP-1, and AM receptor proteins (calcitonin receptor-like receptor [CRLR]) and receptor activity-modifying protein (RAMP2 and RAMP3) mRNA were quantified using real-time PCR (ABI PRISM Sequence Detection System 7000, Applied Biosystems Group) or semi-quantitative RT-PCR after reverse transcription. Immunoreactive AM was measured by a radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA, USA).

NR8383 rat macrophages and human monocytic cell line THP-1 were studied. THP-1 cells were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 72 hours to induce differentiation into macrophage-like cells. The cells were stimulated by LPS (10 ng/mL) and AMBP-1 (500 nM) in the presence or absence of AM or AM receptor antagonists (AM₂₂₋₅₂ or CGRP₈₋₃₇) for 6 and 24 hours. Concentrations of TNF- α , IL-6, and IL-10 in the culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA, R&D Systems, USA). To study desensitisation of the AM receptor, cells were pre-incubated with AM (1 to 1000 nM) for 2 hours followed by a washout period and a re-stimulation by LPS with or without AM/AMBP-1. The concentration of TNF- α , IL-6, and IL-10 in the supernatant was measured.

NR8383 macrophages were stimulated by LPS (10 ng/mL) with or without addition of AM, forskolin (an adenylyl cyclase activator), SQ22536 (Qbiogene, an adenylyl cyclase inhibitor) or dibutyryl-cAMP (a membrane permeable cAMP analogue) or a signalling pathway inhibitor for 6 and 24 hours. Inhibitors of protein tyrosine kinase (PTK) (genistein, Merck, USA), p38 and p42/44 MAPK, protein kinase A (PKA), and protein kinase C (PKC) were used. TNF- α , IL-6, and IL-10 in the supernatant were measured.

Changes in cytokine levels were analysed using

analysis of variance or t-test as appropriate. A P value of <0.05 was considered significant.

Results

Expression of AM, AMBP-1, and AM receptor proteins in the LPS-induced inflammatory response in a rat alveolar macrophage cell line

In unstimulated NR8383 cells, AMBP-1 was constitutively expressed, with a RT-PCR cycle threshold of 26.62 ± 0.08 compared with 18.35 ± 0.07 for β -actin. Basal mRNA expression of CRLR and RAMP-2 were low, with a mean RT-PCR cycle threshold of 30.77 ± 0.03 and 35.52 ± 0.07 , respectively, compared with 18.35 ± 0.07 for β -actin.

LPS increased expression of CRLR by 5- to 26-fold at 6 hours ($P < 0.01$), which declined to 5- to 11-fold above basal at 12 hours and to 2- to 4-fold above basal at 24 hours. IFN- γ , TNF- α , IL-6, or IL-10 had no effect on CRLR mRNA level in unstimulated NR8383 cells. In 10 ng/mL LPS-stimulated cells, IL-10 and IFN- γ reduced CRLR mRNA level at 6 hours from 12.2-fold basal level to 8.0-fold and 5.6-fold, respectively ($P < 0.05$), whereas IL-6 increased the CRLR mRNA level to 16.9-fold ($P < 0.05$). LPS reduced RAMP-2 expression to 0.35-fold basal level at 6 and 12 hours.

AMBP-1 expression was not altered by LPS, but IFN- γ significantly reduced AMBP-1 mRNA level in unstimulated and LPS-stimulated cells to 0.66-fold and 0.47-fold of basal level, respectively, at 6 hours (Fig 1).

IFN- γ at 10 ng/mL increased AM concentration in the culture medium from 12.9 ± 2.3 fmol/mL to 215.9 ± 35.7 fmol/mL ($P < 0.05$), whereas addition of IFN- γ to LPS-stimulated cells increased AM concentration from 120.6 ± 23.8 fmol/mL to 329.7 ± 58.4 fmol/mL ($P < 0.05$).

Effect of AM and AMBP-1 on inflammatory cytokine production in NR8383 and PMA-activated THP-1 cells

AMBP-1 at 500 nM did not alter the concentration of TNF- α , IL-6, or IL-10 in LP-stimulated macrophages. IL-10 secretion from unstimulated NR8383 cells was undetectable and not increased by AM. LPS stimulated IL-10 production that was augmented by 100 nM AM (Fig 2a).

PCR products of IL-10 and TBP are shown (Fig 2b). IL-10 mRNA expression was significantly up-regulated at 6 hours after LPS, paralleled the IL-10 peptide levels and further increased by 140%, 120%, and 65%, with addition of exogenous AM for LPS at 1, 5, and 100 ng/mL, respectively (Fig 2c). LPS-induced IL-10 production was also increased by TNF- α and IL-6 by 71% and 70%, respectively, but was markedly reduced by IFN- γ by 82% (Fig 2d).

In PMA-activated THP-1 cells, LPS markedly increased IL-10 production from as low as 1 ng/mL. 100 nM AM increased LPS-induced IL-10 production by 69% to 112% while IFN- γ reduced it by 77%.

The AM receptor antagonists (AM₂₂₋₅₂ and CGRP₈₋₃₇) did not significantly alter the concentration of TNF- α , IL-6, or IL-10. Pre-incubation with AM followed by washout had no effect on TNF- α , IL-6, or IL-10 production, compared with LPS stimulation alone.

Role of second messenger-dependent kinases in the regulation of AM expression and AM-induced cytokine responses in LPS-stimulated macrophages

Dibutyryl-cAMP (10 μ M) and forskolin (1 μ M) reduced TNF- α production by 50% and increased the production of IL-6 and IL-10 by 105% to 128%. Dibutyryl-cAMP also increased AM concentration from 120.6 \pm 23.8 fmol/mL to 514.5 \pm 102.6 fmol/mL (P <0.05). SQ22536 had no effect on IL-6, IL-10, or AM concentration.

SB203580 inhibited LPS-induced IL-10 production by 56%; the production was partially reversed by exogenous AM and IL-6 but not TNF- α (Fig 3a). In contrast, PD98059 did not significantly affect IL-10 production that could be increased by adding AM or IL-6 (Fig 3b). SB203580 reduced IL-6 production by 13% to 29%; the production was completely reversed by adding AM or TNF- α (Fig 3c). PD98059 reduced IL-6 production by 22% to 26%; the production was reversed by AM but not TNF- α (Fig 3d).

In PMA-activated human THP-1 cells, PD98059 had no effect on LPS-induced IL-10 production but SB203580 inhibited it by 66%. This blockade was not reversible by AM, TNF- α , or IL-6.

PKA-inhibitor did not significantly affect IL-6 and IL-10 production. PKC-inhibitor reduced IL-6 production by 32% (P <0.05), but did not alter IL-10 production. Genistein reduced both IL-6 and IL-10 production by 87%. IL-6 production was partially restored by exogenous AM, but IL-10 production was not rescued by AM, TNF- α , or IL-6.

Discussion

In this study, LPS increased the initial expression of CRLR but reduced the expression of RAMP-2. Expression of CRLR in LPS-stimulated cells was attenuated by IL-10. This might suggest a negative feedback or dampening effect of IL-10 on CRLR expression. IFN- γ increased AM production in LPS-stimulated cells, while it reduced both CRLR and AMBP-1 expression.

In this study, AM significantly augmented LPS-induced IL-10 production in both NR8383 alveolar

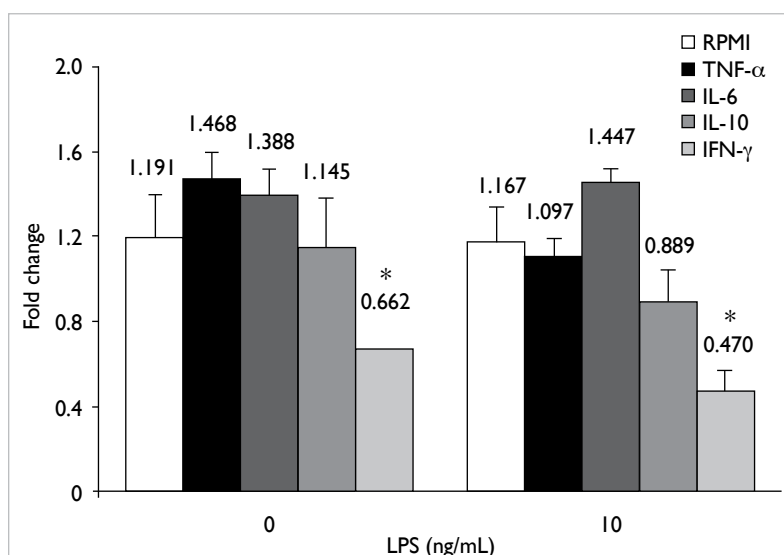


FIG 1. Relative expression of adrenomedullin-binding protein-1 (AMBP-1) in NR8383 cells in response to lipopolysaccharide (LPS) and inflammatory mediators at 6 hours

NR8383 cells were stimulated by different inflammatory mediators in the presence or absence of LPS. Expressions of AMBP-1 mRNA were measured by real-time PCR at 6 hours of incubation

* P <0.05, vs no inflammatory mediator

macrophages and PMA-activated THP-1 cells. This indicates a role of AM production in macrophages and 'macrophage-like' cells. On the contrary, AM receptor antagonists had no significant effect on the production of these inflammatory cytokines on LPS-stimulated NR8383 cells. These indicate that other mediators or alternative pathways independent of AM may be sufficient to stimulate the IL-10 production in these cells. Furthermore, IFN- γ reduced LPS-induced IL-10 production in both NR8383 alveolar macrophages and PMA-activated THP-1 cells that might be related to its inhibitory effect on the AM receptor and AMBP-1 expression. Thus, AM and IFN- γ might have opposite roles in the regulation of the anti-inflammatory cytokine IL-10 in both rat and human macrophages.

In this study, cAMP mediated the increased expression of AM, IL-6 and IL-10, and the decreased expression of TNF- α following LPS stimulation. Moreover, the production of IL-6 was mediated by p38-MAPK, p42/44-MAPK, PKC, and PTK, whereas that of IL-10 was mediated by p38-MAPK and PTK. Activation of the p42/44- and p38-MAPK pathways is common early signals necessary for LPS-induced cytokine production in monocytes and macrophages.⁷

In this study, blockade of the p38-MAPK pathway by SB203580 could be reversed by exogenous AM and IL-6, suggesting that other

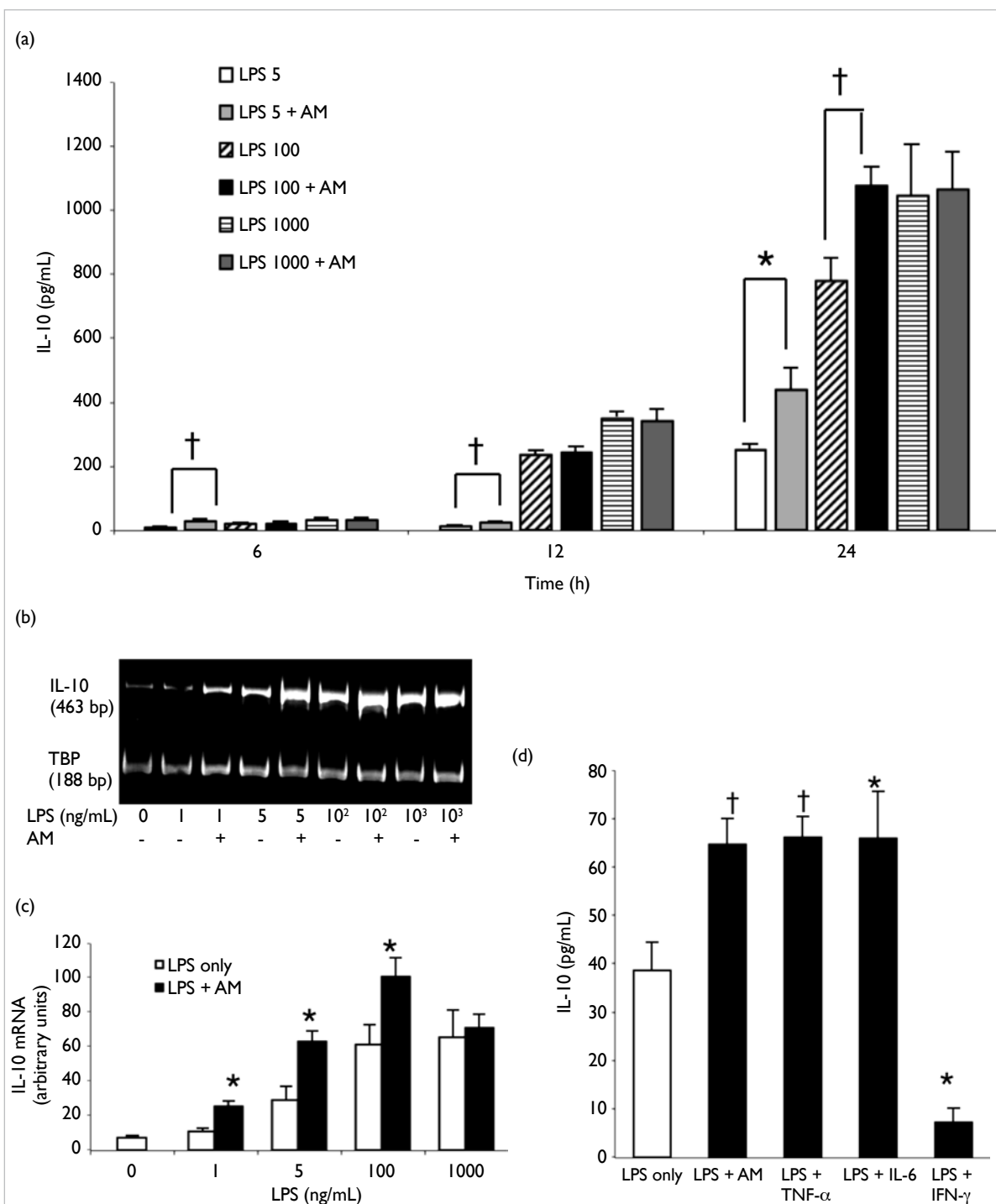


FIG 2. Effect of adrenomedullin (AM) and inflammatory mediators on lipopolysaccharide (LPS)-induced IL-10 production in NR8383 cells

(a) NR8383 cells were stimulated with LPS in the presence or absence of exogenous AM at 100 nM. IL-10 concentrations in the culture media were measured at 6, 12, and 24 hours by ELISA. (b) Representative results of gene-specific PCR products of IL-10 and TBP mRNA at 6 hours of incubation analysed by gel electrophoresis. (c) Semi-quantitative RT-PCR analysis was performed to determine IL-10 mRNA expression after normalising with TBP mRNA level at 6 hours after LPS-stimulation. (d) NR8383 cells were stimulated with 1 ng/mL of LPS in the presence or absence of AM (at 100 nM) or TNF- α , IL-6, and IFN- γ (each at 30 ng/mL). IL-10 concentrations were measured at 24 hours of incubation

* P < 0.05, vs LPS alone

† P < 0.01, vs LPS alone

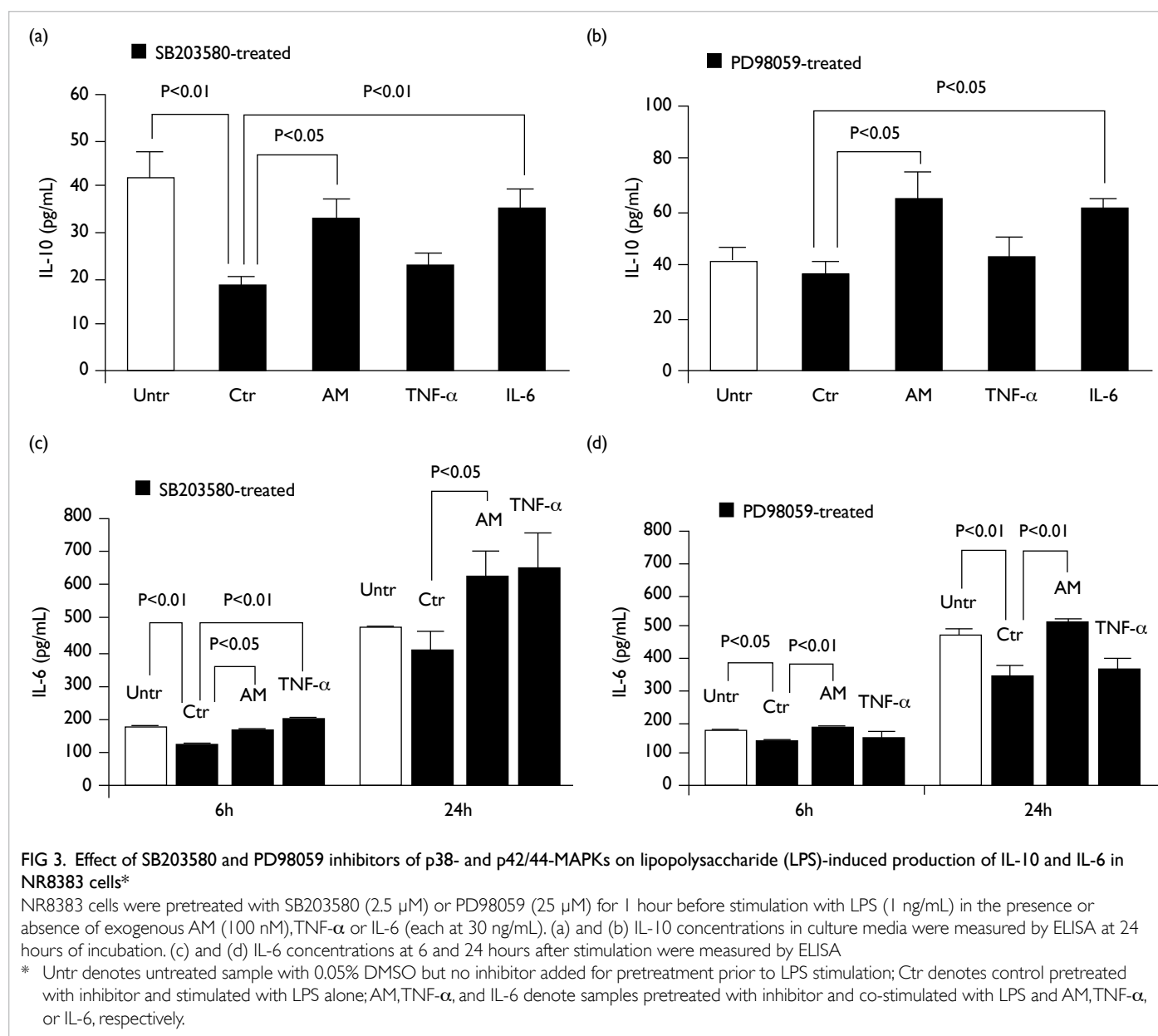


FIG 3. Effect of SB203580 and PD98059 inhibitors of p38- and p42/44-MAPKs on lipopolysaccharide (LPS)-induced production of IL-10 and IL-6 in NR8383 cells*

NR8383 cells were pretreated with SB203580 (2.5 μ M) or PD98059 (25 μ M) for 1 hour before stimulation with LPS (1 ng/mL) in the presence or absence of exogenous AM (100 nM), TNF- α or IL-6 (each at 30 ng/mL). (a) and (b) IL-10 concentrations in culture media were measured by ELISA at 24 hours of incubation. (c) and (d) IL-6 concentrations at 6 and 24 hours after stimulation were measured by ELISA

* Untr denotes untreated sample with 0.05% DMSO but no inhibitor added for pretreatment prior to LPS stimulation; Ctr denotes control pretreated with inhibitor and stimulated with LPS alone; AM, TNF- α , and IL-6 denote samples pretreated with inhibitor and co-stimulated with LPS and AM, TNF- α , or IL-6, respectively.

pathways may exist to induce IL-10 production upon LPS-stimulation. LPS-induced IL-10 and IL-6 production were markedly reduced by genistein, indicating that PTK-mediated pathways play an important role. The fact that IL-10 synthesis could not be rescued by any of the mediators that increase IL-10 production indicates a decisive role of PTKs in IL-10 production in this macrophage cell line. In contrast, IL-6 production could be partially rescued by AM in the presence of genistein, suggesting that AM activates more than one pathway to induce IL-6 production.

Conclusions

IFN- γ increases the expression of AM but

downregulates CRLR and AMBP-1. AM augments LPS-induced IL-10, independently of increases in TNF- α and IL-6, and acts in opposition to IFN- γ . cAMP mediates the increased expression of AM, IL-6, and IL-10, and the decreased expression of TNF- α following LPS stimulation. The production of IL-6 is mediated by p38-MAPK, p42/44-MAPK, PKC and PTK, whereas that of IL-10 is mediated by p38-MAPK and PTK. AM plays a role in the regulation of inflammation. It augments the production of IL-10, a cytokine that limits inflammation. Nonetheless, the effectiveness of AM, although elevated in inflammation, may be diminished by IFN- γ that down-regulates AM's receptor and its binding protein. The downstream pathways that

mediate IL-10 and IL-6 production are identified. These findings suggest new targets for therapeutic intervention.

Acknowledgements

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#05050082). Vivian Chan and Garry Chang contributed to the project as research assistants.

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